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FGF signaling regulates Wnt ligand expression to control vulval cell lineage polarity in *C. elegans*

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SUMMARY

The interpretation of extracellular cues leading to the polarization of intracellular components and asymmetric cell divisions is a fundamental part of metazoan organogenesis. The *Caenorhabditis elegans* vulva, with its invariant cell lineage and interaction of multiple cell signaling pathways, provides an excellent model for the study of cell polarity within an organized epithelial tissue. Here, we show that the fibroblast growth factor (FGF) pathway acts in concert with the Frizzled homolog LIN-17 to influence the localization of SYS-1, a component of the Wnt/β-catenin asymmetry pathway, indirectly through the regulation of *cwn-1*. The source of the FGF ligand is the primary vulval precursor cell (VPC) P6.p, which controls the orientation of the neighboring secondary VPC P7.p by signaling through the sex myoblasts (SMs), activating the FGF pathway. The Wnt CWN-1 is expressed in the posterior body wall muscle of the worm as well as in the SMs, making it the only Wnt expressed on the posterior and anterior sides of P7.p at the time of the polarity decision. Both sources of *cwn-1* act instructively to influence P7.p polarity in the direction of the highest Wnt signal. Using single molecule fluorescence *in situ* hybridization, we show that the FGF pathway regulates the expression of *cwn-1* in the SMs. These results demonstrate an interaction between FGF and Wnt in *C. elegans* development and vulval cell lineage polarity, and highlight the promiscuous nature of Wnts and the importance of Wnt gradient directionality within *C. elegans*.

KEY WORDS: FGF, Wnt, *C. elegans*, Vulval development, Cell polarity

INTRODUCTION

The orientation of asymmetric cell divisions is essential for proper tissue architecture and organogenesis (Strutt, 2005). Loss of cell polarity and asymmetry is a major factor in tumor formation, and growing evidence illustrates its importance in understanding human cancer (Wodarz and Näthke, 2007). Because polarity and asymmetry are such vital components of proper organ formation, cell-cell interactions involving crosstalk between multiple signaling pathways are often incorporated to regulate these processes tightly. The *Caenorhabditis elegans* vulva provides a simple model in which to study this phenomenon owing to the small number of cells, invariant cell lineage and developmental timing, and cell signaling mechanisms involved within vulval formation (reviewed by Sternberg, 2005; reviewed by Gupta et al., 2012). Here, we examine the interaction of FGF and Wnt signaling in controlling vulval cell lineage orientation.

The *C. elegans* vulva is formed from divisions of three VPCs, P5.p, P6.p and P7.p, arranged along the anterior-posterior axis in the ventral epithelium (Sulston and Horvitz, 1977). During the L3 (third larval) stage, a combination of epidermal growth factor (EGF), Notch and Wnt signals instructs the VPCs to adopt fates corresponding to particular lineage patterns. P6.p adopts a primary fate and undergoes three rounds of symmetric divisions that lead to eight cells that form the vulval lumen. P5.p and P7.p adopt the secondary fate, which leads to three rounds of asymmetric cell divisions forming seven cells that create the anterior and posterior sides of the vulva (Fig. 1). The outermost progeny of P5.p and P7.p adhere to the epidermis whereas the innermost progeny join the descendants of P6.p in forming the vulval lumen. The descendants of P5.p and P7.p display mirror symmetry about the center of the vulva.

Previous analyses show that the orientation of P5.p and P7.p descendants is determined by the interaction of multiple Wnt signals. In the absence of all Wnts, the VPCs display a randomized orientation, which is likely to be the default (Green et al., 2008) (Fig. 1). Two separate Wnts from the anchor cell, LIN-44 and MOM-2 acting through receptors LIN-17/Frizzled and LIN-18/Ryk, respectively, regulate P7.p orientation (Ferguson et al., 1987; Sternberg and Horvitz, 1988; Sawa et al., 1996; Inoue et al., 2004; Gleason et al., 2006). In the absence of these signals, the orientation of the progeny of P7.p mimic those of P5.p and face towards the posterior of the worm, a phenotype referred to as posterior-reversed vulval lineage (P-Rvl; Fig. 2). This posterior orientation is dependent on the instructive signal of EGL-20, a Wnt expressed in the tail acting through CAM-1/ROR and VANG-1/Van Gogh, and is referred to as 'ground polarity'. In response to the Wnt signals from the anchor cell, LIN-17 and LIN-18 orient P7.p to face the center. This reorientation is described as 'refined polarity' and is the wild-type orientation (Green et al., 2008) (Fig. 1).

The adult vulva is essential for egg laying and mating. The sex muscles, consisting of uterine and vulval muscles, are required for egg laying. The vulval muscles are formed from the migrating SMs (Thomas et al., 1990). Both gonad-independent and -dependent pathways control the anterior migration of SMs in the *C. elegans* hermaphrodite (Burdine et al., 1998; Branda and Stern, 2000). EGL-17/FGF is the gonad-dependent attractant and acts via the FGF receptor EGL-15. The dorsal uterus, ventral uterus, anchor cell and P6.p produce the gonad-dependent attractant (Branda and Stern, 2000). The function of EGL-17 in SM migration requires other components of the FGF pathway; genetic mutations of each component affect the migration and final location of the SMs (Sundaram et al., 1996). Because *egl-17* expression in P6.p is not

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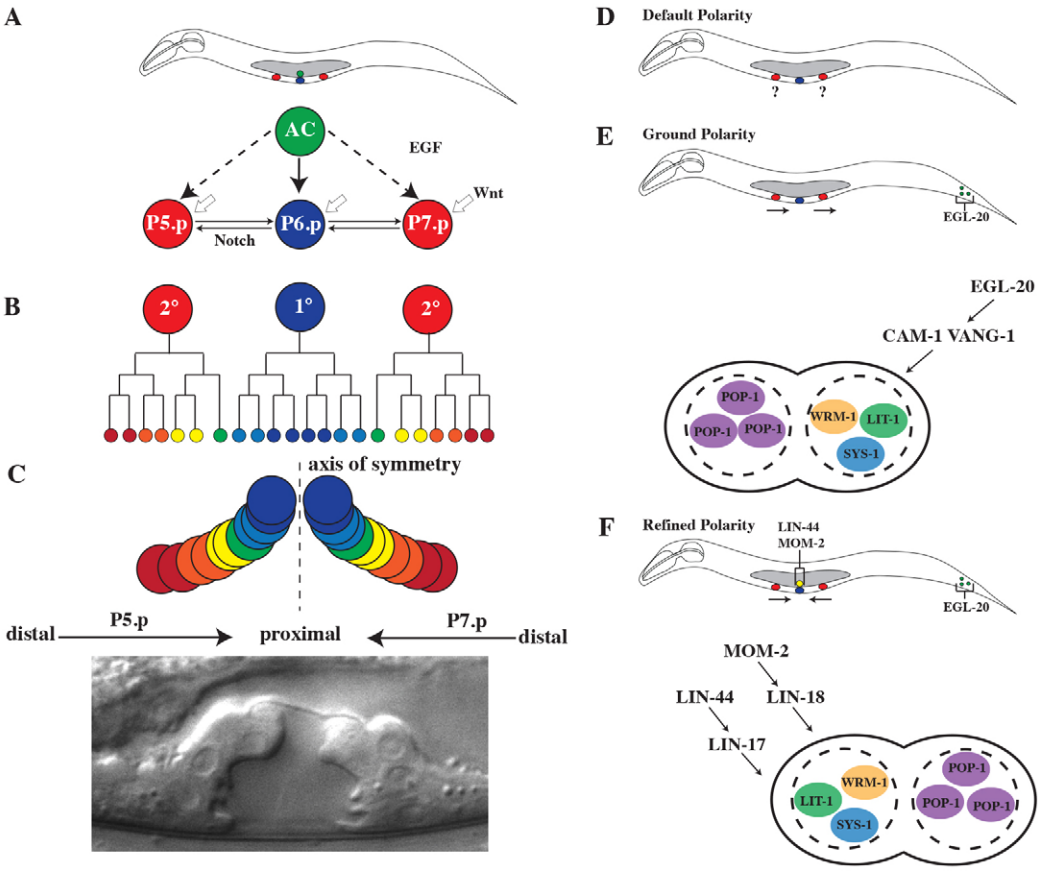

Fig. 1. *C. elegans* vulval development. (**A**) Schematic of vulval induction illustrating sources of EGF, Notch and Wnt. AC, anchor cell. (**B**) Lineage trees of VPC progeny: P5.p, secondary (2°) fate on the left; P6.p, primary (1°) fate in center; and P7.p, secondary 2° fate on the right. The progeny of each cell is color coded: A cells, red; B cells (B1 and B2), orange; C cells, yellow; D cells, green; E cells, light blue; F cells, dark blue. (**C**) Final conformation of vulval cells shown as a cartoon and Nomarski image in mid-L4 stage. Mirror symmetry is noted about the vulval center. Proximal daughter cells of P5.p and P7.p join the daughters of P6.p in forming the vulval lumen, whereas the distal most daughters of P5.p and P7.p adhere to the ventral epidermis. (**D**) The default polarity of P5.p and P7.p is random in the absence of all Wnts. (**E**) EGL-20 is expressed in the tail (green circles) and establishes ground polarity in which both P5.p and P7.p face the posterior as a result of asymmetric localization of SYS-1, LIT-1 and WRM-1 to the posterior daughter of P7.p and POP-1 to the anterior daughter. (**F**) LIN-44 and MOM-2 are expressed in the anchor cell (yellow circle) resulting in refined polarity in which P5.p and P7.p both face towards the center as a result of asymmetric localization of SYS-1, LIT-1 and WRM-1 to the anterior daughter cell of P7.p and POP-1 to the posterior daughter cell.

necessary, but is sufficient, for proper SM migration, it is believed that this expression is used to fine-tune the gonadal attraction (Burdine et al., 1998). *egl-17* expression in P6.p is activated by the inductive signal from the anchor cell that occurs in early L3, at which time the SMs have reached the center of the gonad (Fig. 3).

Interactions between Wnt and other signaling pathways during vulval orientation have not been explored. Here, we present evidence that FGF signaling promotes the wild-type orientation of P7.p. We show that FGF signaling interacts genetically with LIN-17 and indirectly controls the localization of SYS-1/β-catenin, a key component of Wnt/β-catenin asymmetry pathway. The primary cell, P6.p, is the source of the EGL-17 signal that controls polarity and acts through EGL-15 and the remainder of the FGF pathway in the migrating SMs. The effect of FGF signaling on vulval orientation is two-sided. First, the SMs must reach their final position, around the gonad center, then EGL-17 must activate the remainder of the FGF pathway in the SMs. Using single molecule fluorescence *in situ* hybridization (smFISH), we discovered that the FGF pathway is necessary for the regulation of a Wnt, *cwn-1*, in the left and right SMs as they flank the center of the gonad during the polarity decision of P7.p. *cwn-1* is also expressed strongly in the posterior body wall muscle, making it the only Wnt with sources of expression on both the anterior and posterior sides of P7.p. We demonstrate that these two sources act instructively and add to the overall Wnt gradient in both the anterior- and posterior-directing pathways.

MATERIALS AND METHODS

Strains and genetics

C. elegans was handled as described previously (Brenner, 1974). All strains used (listed in supplementary material Table S1) are derivatives of *C. elegans* N2 Bristol strain. The alleles used are as follows. LGI: *lin-17(n671)*, *sem-2(n1343)*. LGII: *cwn-1(ok546)*, *ayIs4[egl-17::gfp, dpy-20(+)]*. LGIII: *qIs95[pSYS-1::VNS::SYS-1* with *pttx-3::dsRed]*. LGIV: *lin-45(sy96)*, *dpy-20(e1282)*. LGX: *lin-18(e620)*, *egl-17(e1313)*, *egl-17(n1377)*, *egl-15(n484)*, *sem-5(n1779, cs15, n2109, n2195)*, *ksr-1(ku68)*. The strain *ayIs4[egl-17::GFP, dpy-20(+)]; dpy-20(e1282); lin-18(e620)* was constructed by crossing strains NH2466 with CB620 (Ferguson and Horvitz, 1985; Burdine et al., 1998). For RNAi

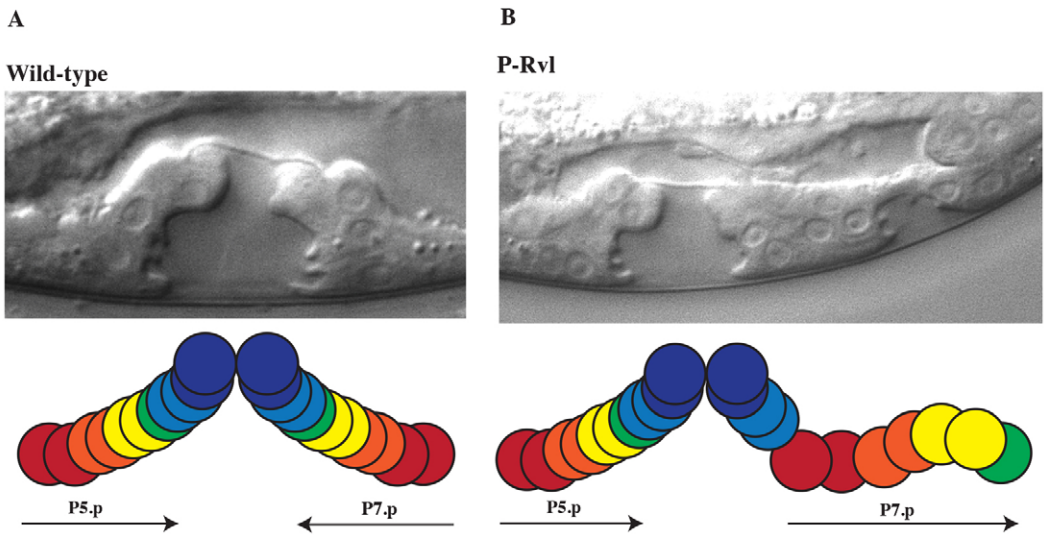

Fig. 2. Wild-type vulva versus posterior-reversed vulval lineage vulva. (**A**) Wild-type vulva formed from 22 progeny of three VPCs: P5.p, P6.p and P7.p. The progeny of P5.p and P7.p form mirror symmetry about the vulval center. (**B**) Posterior-reversed vulval lineage: the daughter cells of P7.p mimic those of P5.p. Both images taken with *sem-5(n1779)* background. Color key as described for Fig. 1.

experiments, gravid hermaphrodites were fed RNAi-expressing bacteria and their L4 progeny were scored.

Scoring vulval phenotypes

To classify the vulval phenotype as wild type or P-Rvl, animals were scored in the mid-L4 stage. Animals were classified as P-Rvl if the primary and secondary VPCs were induced but separated by adherent cells (Katz et al., 1995). Only fully induced vulvae were scored.

Transgenics

To make the *CWN-1::GFP* construct backbone, *cwn-1* was amplified from genomic DNA (forward primer, ATGTGATGTCGACAAAAATGCTGAAATCTACACAAGTGATCC; reverse primer, GCAGCTTCTAGATAAGCATAAATACTTCTCAATTCG) and inserted into Fire vector pPD95.75 using restriction sites *Sal*I and *Xba*I. To create *Pegl-17::CWN-1::GFP*, first the promoter region of *egl-17* was amplified from genomic DNA (forward primer, GCCTATGCAGCATTGGAGGATG; reverse primer, GGATCACTTGTGTAGATTTCAGCATAGCTCACATTTCGGGCACCTG). The promoter region of *egl-17* was then fused to *CWN-1::GFP* (forward primer, GCCTATGCAGCATTGGAGGATG; reverse primer, AAGGGCCCGTACGGCCGACTA) (Hobert, 2002). The *Pegl-17::CWN-1::GFP* extrachromosomal array was generated by creating an injection mix consisting of 1 ng/μl *Pegl-17::CWN-1::GFP*, 7 ng/μl *Pmyo-2::dsRed* and 142 ng/μl DNA ladder and injecting the mix into *cwn-1(ok546); lin-18(e620)* as well as *lin-18(e620) egl-15(n484)* animals as described (Mello et al., 1991).

Ablations

Cell ablation experiments were performed as described (Bargmann and Avery, 1995). P6.p was ablated post-induction, but before the first division of the VPCs. Strain NH2466 was crossed into *lin-18(e620)* in order to time accurately the experiments by monitoring *egl-17* expression in P6.p. The M cell was ablated in the early L1 stage in both a *lin-18(e620)* as well as a *cwn-1(ok546); lin-18(e620)* background. After ablations, the animals were recovered from slides and grown at 20°C until the mid-L4 stage when the vulval phenotype could be scored. Mock ablations were performed by placing appropriately staged worms on a slide for ~10 minutes, recovering them, and then scoring their vulval phenotype in mid-L4.

Single molecule mRNA FISH

Probes for *cwn-1* detection were provided as a gift from Dong hyun Kim (Harterink et al., 2011). Preparation and hybridization steps were performed as previously described (Raj et al., 2008). Both strains, N2 and *egl-15(n484)*, were prepared and imaged in an identical manner. Multiple plates were grown until full of gravid hermaphrodites and then bleached. The eggs from these bleachings were placed on fresh plates and grown at 20°C to enable an approximate synchronization of animals. After the animals had reached vulval induction they were washed from the plates using ddH_2O and fixed in 3.7% formaldehyde in 1× PBS for 1 hour. Fixed animals were then permeabilized in 70% ethanol for 48 hours. Animals were washed and the *cwn-1* probes coupled with Cy5 were added and left overnight at 37°C. The next day, animals were washed and DAPI stained. Images were taken in *z*-stacks using an Olympus IX2-UCB microscope, Andor iKon-M 934 camera, and appropriate optical filters for Cy5 and DAPI. *z*-stacks were flattened into single images using Fiji. Quantification of single mRNA transcripts within the SMs was performed using a MATLAB script and manually corrected for further accuracy.

RESULTS

FGF signaling defects enhance the *lin-18* phenotype

The *C. elegans* Grb2 ortholog *sem-5* acts in both vulval induction, controlled by the EGF pathway, and SM migration, controlled by the FGF pathway (Clark et al., 1992; reviewed by Sundaram, 2006). SEM-5 is an adaptor protein the SH2 domain of which probably binds to the phospho-tyrosine residues of LET-23/EGFR and EGL-15/FGFR and recruits the RAS exchange factor SOS-1/Son of sevenless via its SH3 domains. Expression of the FGF ligand in P6.p is dependent upon vulval induction (Burdine et al., 1998).

Different alleles of *sem-5* have varying degrees of effect on vulval induction as well as SM migration, but a role in vulval orientation has not previously been reported. We scored the vulval lineage of P7.p in four different alleles of *sem-5*. Two alleles, *n2019* and *cs15*, which cause a glycine to alanine substitution in the first SH3 domain and an opal stop in the second SH3 domain, respectively, cause polarity and induction defects, whereas *n2195*, which causes a glycine to arginine substitution in the second SH3 domain, yields neither polarity nor induction defects. The fourth allele, *n1779*, which causes a glutamate to lysine substitution in the SH2 domain, results in a 13% P-Rvl phenotype, affecting polarity, but not induction (Table 1). We thus used *sem-5(n1779)* as the canonical

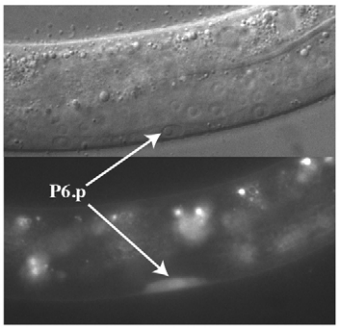

Fig. 3. *egl-17::gfp* expression in P6.p. *egl-17* is activated by EGF signaling and is expressed in P6.p. Expression of *egl-17* is used as a marker for vulval induction.

allele. Previously known components involved in the regulation of vulval cell lineage polarity include LIN-17, LIN-18, CAM-1 and VANG-1, all of which are Wnt signaling components (Inoue et al., 2004; Gleason et al., 2006; Green et al., 2008). SEM-5 is the first non-Wnt signaling component found to be involved in vulval orientation.

We next looked at the involvement of each component in the FGF pathway. No allele of *egl-17*, *egl-15* or any other downstream FGF component other than *sem-5* had any effect on orientation as single mutants (Table 1), which is probably due to the involvement of *sem-5* in one of the other pathways controlling vulval orientation as well as its role in the FGF pathway. No null mutations of the downstream components of the FGF pathway are available owing to their lack of viability. There are conflicting reports on whether *egl-17(n1377)* is a null or a reduced-function allele, though owing to the severity of its phenotype as well as the frequency with which *egl-17* mutations arise in ethyl methanesulfonate (EMS) screens, it is usually considered null (Burdine et al., 1997; Château et al., 2010).

Table 1. FGF signaling enhances the P-Rvl phenotype of *lin-18(e620)*

Genotype	% P-Rvl	*n*	*P*-value
N2	0	100	
lin-17(n671)	74	100	
lin-18(e620)	31	100	
lin-17(n671); lin-18(e620)	100	40	
egl-17(n1377)	0	40	
egl-17(e1313)	0	36	
egl-15(n484)	0	100	
sem-5(n1779)	13	45	
sem-5(n2019)	33	18	
sem-5(cs15)	7	30	
sem-5(n2195)	0	30	
sos-1(RNAi)	0	30	
let-60(RNAi)	0	30	
lin-45(sy96)	0	35	
mek-2(RNAi)	0	30	
mpk-1(RNAi)	0	30	
ksr-1(ku68)	0	30	
lin-17(n671); egl-15(n484)	71	31	
lin-18(e620) egl-15(n484)	63	52	0.0001
lin-18(e620) egl-15(RNAi)	63	32	0.0031
egl-17(e1313) lin-18(e620)	54	48	0.0111
egl-17(n1377) lin-18(e620)	57	30	0.0168
lin-18(e620) sem-5(n1779)	57	30	0.0168
lin-18(e620) sos-1(RNAi)	63	30	0.0024
lin-18(e620) let-60(RNAi)	68	31	0.0006
lin-18(e620) lin-45(sy96)	60	30	0.0054
lin-18(e620) mek-2(RNAi)	67	30	0.0006
lin-18(e620) mpk-1(RNAi)	68	34	0.0002
ksr-1(ku68) lin-18(e620)	66	35	0.0005

Double mutants were constructed between *lin-18(e620)* and each known component of the FGF pathway. Vulval phenotypes were scored during mid-L4. *P*-values were calculated in comparison with *lin-18(e620)* using Fisher's exact test.

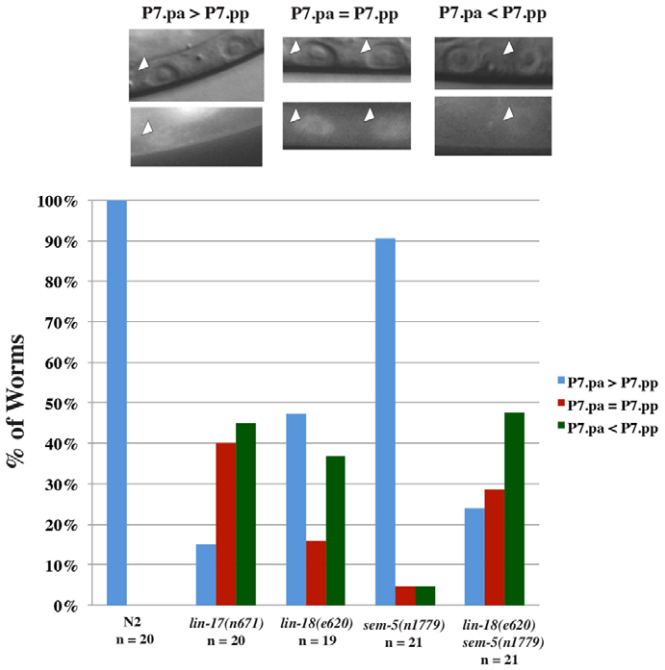

Fig. 4. Subcellular localization of VNS::SYS-1. The localization pattern of VNS::SYS-1 in P7.p daughter cells. The resulting pattern was classified by eye into three categories: SYS-1 enriched in the anterior daughter (P7.pa >P7.pp), SYS-1 present at similar levels in both daughters (P7.pa=P7.pp), and SYS-1 enriched in the anterior daughter (P7.pa <P7.pp). A representative image of each scenario is shown. Graph shows percentage of animals with the pattern of SYS-1 localization indicated.

To understand the genetic relationship between FGF signaling and the previously known Wnt polarity pathway components required for the wild-type vulval orientation, we constructed double mutants of *egl-15(n484)* with the canonical null alleles of *lin-17* and *lin-18* (Table 1). Because *egl-15(n484)* enhances the *lin-18(e620)* P-Rvl phenotype from 31 to 63% and has no effect on *lin-17(n671)*, we believe the FGF pathway is working with the LIN-17 pathway to control vulval orientation. To test this hypothesis, we constructed double mutants of all known FGF pathway components with *lin-18(e620)* or used RNAi in a *lin-18(e620)* background (Table 1). Alleles of *egl-17* enhanced *lin-18(e620)* to ~55% P-Rvl, similar to the effect of *sem-5(n1779)*, which enhanced *lin-18(e620)* to 57% P-Rvl. The double mutant with the Son of sevenless ortholog *sos-1* had a P-Rvl of 63%, whereas the double mutant with the Ras ortholog *let-60* enhanced the *lin-18(e620)* phenotype to 68% P-Rvl. Finally, the MAP kinase cascade consisting of *lin-45*, *mek-2*, *mpk-1* and the scaffold *ksr-1*, also enhanced the vulval phenotype to 60, 67, 68 and 66% P-Rvl, respectively. Each component of the pathway enhanced the P-Rvl phenotype of *lin-18(e620)* to roughly the same degree, implying that the entire FGF pathway functions together. This pathway is likely to act with LIN-17 as the mutations enhance *lin-18(lf)* but not *lin-17(lf)* alleles. If FGF signaling was working separately from the LIN-17 pathway, we would expect

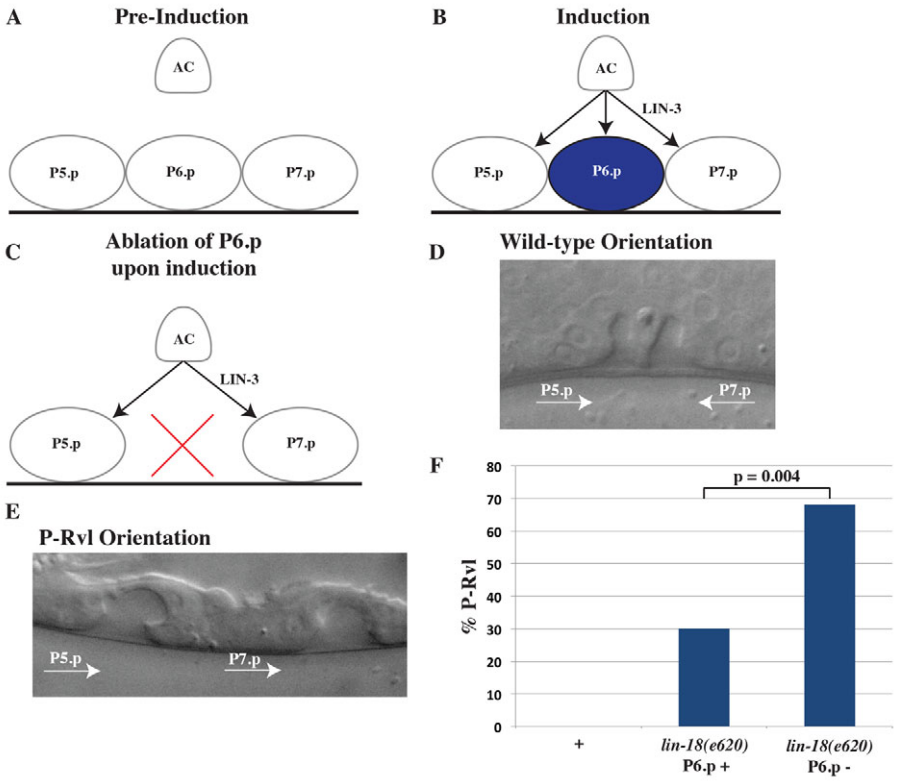

Fig. 5. P6.p influences the polarity of P7.p. (**A**) Prior to induction, the anchor cell (AC) is directly dorsal to P6.p. (**B**) During induction, the anchor cell produces LIN-3, which is supplied to P5-7.p. Induction activates *egl-17*, illustrated in blue, within P6.p. (**C**) P6.p is ablated at the start of induction, but prior to the VPC polarity choice, leaving only P5.p and P7.p, the secondary VPCs. (**D**) Wild-type orientation of a worm with P6.p ablated. (**E**). P-Rvl orientation of a worm with P6.p ablated. (**F**) Ablating P6.p enhances the phenotype of *lin-18(e620)*.

FGF to enhance the *lin-17(lf)* phenotype as it does *lin-18(lf)*; however, because there is no effect on *lin-17(lf)* we assume that FGF acts in concert with, not separately from, LIN-17.

FGF regulates the localization of SYS-1/β-catenin

The polarity of the P7.p cell divisions is controlled by the Wnt/β-catenin asymmetry pathway (Green et al., 2008), which includes the β-catenin-like proteins SYS-1 and WRM-1, POP-1/TCF, and the Nemo-like-kinase LIT-1 (reviewed by Mizumoto and Sawa, 2007). The Wnt/β-catenin asymmetry pathway ensures different ratios of SYS-1 to POP-1, controlling the differential transcription of Wnt target genes between daughters of an asymmetric cell division. Because our genetic data show an interaction between FGF and LIN-17, we wanted to determine whether the FGF pathway, like LIN-17, can control the asymmetric localization of proteins between daughter cells of P7.p. The initial establishment of vulval polarity can be observed through the localization of VENUS::SYS-1 (VNS::SYS-1), localized in a high (P7.pa)/low (P7.pp) pattern in the wild-type worm, reciprocal to the localization of POP-1 (Phillips et al., 2007; Green et al., 2008).

As previously reported, VNS::SYS-1 asymmetry in P7.p daughter cells is often lost in *lin-17(n671)* and *lin-18(e620)* mutants (Fig. 4). These mutants display two aberrant patterns of VNS::SYS-1 localization as well as the wild-type pattern, though less frequently. The two deviant localization patterns include one in which both P7.pa and P7.pp express equal amounts of VNS::SYS-1, and a reversed VNS::SYS-1 pattern in which P7.pp is enriched with VNS::SYS-1. By observing VNS::SYS-1 localization in *sem-5(n1779)* mutants we found two out of 20 worms having an atypical localization of VNS::SYS-1, which reflects the small percentage of worms that have P-Rvl phenotype (13% P-Rvl). Because in wild-type worms VNS::SYS-1 invariably localized to the anterior daughter of P7.p, this result is physiologically relevant. In agreement with our model, no other VPCs show defective VNS::SYS-1 localization in a *sem-5(n1779)* background. This observation confirms that FGF pathway controls vulval cell polarity by interacting with LIN-17, and thus the Wnt/β-catenin asymmetry pathway, and indicates that the FGF effect is at the level of P7.p rather than its progeny. Moreover, the reversal of VNS::SYS-1 localization in *lin-18(e620) sem-5(n1779)* double mutants is slightly greater than in *lin-18(e620)* alone (Fig. 4).

P6.p is the source of EGL-17 and controls P7.p polarity

Once it was confirmed that FGF regulates P7.p polarity, we wanted to find the source of FGF. Because the FGF ligand EGL-17 is expressed in the primary VPC P6.p after EGF has activated vulval induction (Burdine et al., 1998; Fig. 3), we hypothesized that P6.p could be the source of the polarity cue. To date, only the anchor cell and the tail of the worm have been shown to be sources of polarity cues; there has been no evidence of the primary cell regulating the polarity of its secondary neighbors despite their crosstalk during vulval induction (Sternberg and Horvitz, 1989; Levitan and Greenwald, 1998). We ablated P6.p after it received its induction cue, but prior to any polarity choice of P7.p. We used a *Pegl-17::gfp* construct to time induction, and ablated the primary cell in both a wild-type background as well as a *lin-18(e620)* background to sensitize the animals to defects in FGF signaling. Worms were monitored until the *Pegl-17::gfp* construct expressed in P6.p and then P6.p was ablated using a laser microbeam (Fig. 5). Similarly to the single mutants of the FGF pathway, ablating P6.p in a wild-type background does not lead to any instances of the P-Rvl phenotype. However, the ablation of P6.p in a *lin-18(e620)* background showed a strong enhancement of the *lin-18(e620)* P-Rvl phenotype, similar to that of every FGF pathway component mutant: the mock-ablated animals had a 30% P-Rvl phenotype whereas the ablated animals

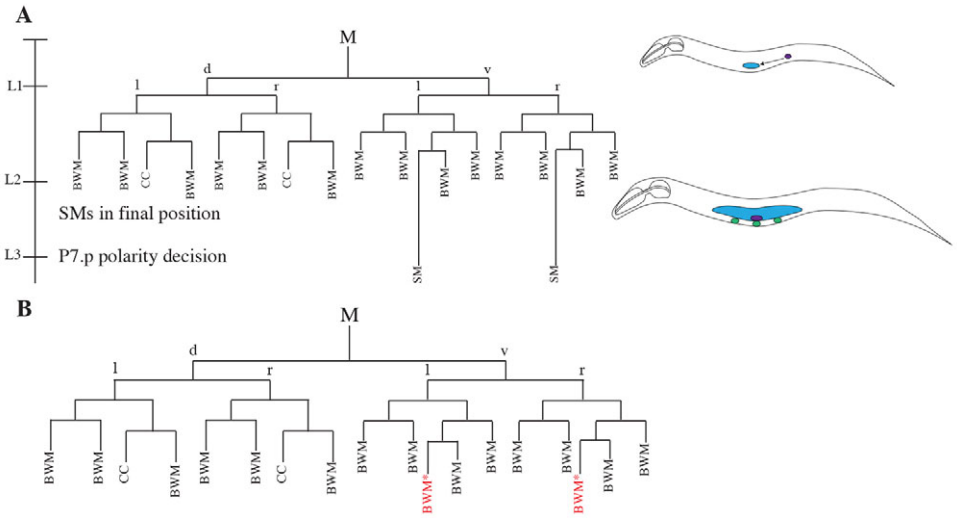

Fig. 6. The sex myoblasts influence the polarity of P7.p. (**A**) The sex myoblasts arise from the M cell as shown in the cell lineage. The sex myoblasts are born 13 hours post-hatch and migrate anteriorly until they reach their final position, flanking the center of the gonad. The polarity decision of P7.p is made after the sex myoblasts have reached their final position and prior to their first division. The M cell/sex myoblasts are shown in purple, the gonad blue and the VPCs green. (**B**) The M cell lineage shown in a *sem-2(n1343)* background. The SMs do not form in this background, but instead become posterior body wall muscle, marked in red and with an asterisk.

had a 68% P-Rvl phenotype. These data suggest that P6.p produces the EGL-17 ligand cue that directs the polarity of P7.p, and the primary vulval cell influences polarity of the neighboring secondary vulval cells.

Ablation of the sex myoblasts enhances the *lin-18* phenotype

After verifying the location of the EGL-17 source, we wanted to confirm the location of the receptor and remainder of the FGF signaling cascade that influences cell orientation. EGL-15 is expressed in the SMs and is necessary for proper SM migration (DeVore et al., 1995; Sundaram et al., 1996; Branda and Stern, 2000; Lo et al., 2008). To determine whether the polarity cue is acting through the SMs or possibly through the VPCs, we examined the expression pattern of *egl-15* using a GFP translational fusion and found no expression in P7.p or any other VPC; however, expression was seen in the M cell lineage, consistent with previous observations (Lo et al., 2008).

The SMs are born from the M cell ~13 hours post-hatching (Sulston and Horvitz, 1977), begin migrating ~2 hours after they form, and reach their final position, flanking the gonad center, 4 hours after beginning migration (Branda and Stern, 2000) (Fig. 6). If the SMs are the source of the FGF polarity pathway we should see an enhancement of the *lin-18(e620)* P-Rvl phenotype; however, if the source is in another location, such as the vulval precursor cells, we would expect to see no enhancement. We ablated the M cell, the precursor to both the left and right SMs, in 29 worms, ~10 hours post-hatching, in a *lin-18(e620)* background. Ablation of the M cell resulted in a strong enhancement of the *lin-18(e620)* phenotype in the same manner as all FGF mutants as well as in the ablation of P6.p: specifically, the M cell-ablated worms showed a 66% P-Rvl phenotype compared with 30% in the non-ablated controls (Table 2).

Because the M cell descendants also contribute to the posterior body wall muscle and coelomocytes, we sought a cleaner way to eliminate the SMs before the polarity cue. The SoxC ortholog *sem-2(n1343)* alters the M cell lineage and prevents the formation of the SMs by driving the cells initially destined to become SMs to become posterior body wall muscle (Tian et al., 2011) (Fig. 6). Constructing *sem-2(n1343); lin-18(e620)* double mutants results in a 68% P-Rvl phenotype, confirming that the SMs influence the polarity choice of P7.p.

We wanted to observe the effect on vulval orientation in a mutant that inhibits SM migration independently of FGF signaling and does not eliminate FGF signaling within the SMs. *mig-2* encodes a member of the Rho family of GTP-binding proteins, is expressed in the SMs, and prevents the SMs from wild-type migration in approximately half of the animals (Forrester and Garriga, 1997; Zipkin et al., 1997; Kishore and Sundaram, 2002). Because half the SMs do not migrate to their final wild-type position, we hypothesized that these SMs would not be capable of giving the polarity cue to P7.p as they do not migrate to the anterior of the cell. *mig-2* RNAi-treated *lin-18(e620)* animals have a 56% P-Rvl phenotype, a significant increase from the *lin-18(e620)* single mutant, confirming that the SMs must migrate to their wild-type position to transmit the polarity cue to P7.p (Table 2).

These results, along with the expression pattern of *egl-15*, indicate that the FGF polarity signal comes from P6.p and requires the SMs. Because the polarity decision of the vulval precursor cells is made prior to anaphase of the first cell division, we believe that the FGF polarity cue acts once the SMs have reached their final position flanking the center of the gonad. Mutations of each component of the FGF pathway have varying degrees of penetrance on the migration of the SMs (Sundaram et al., 1996). By contrast, the effects of these mutants on vulval lineage orientation are strikingly similar. We believe that the effect of FGF signaling on P7.p orientation is two-sided. First, the SMs must migrate to the anterior side of P7.p via an uncompromised FGF signal. Once the SMs have migrated to the anterior side of P7.p, the FGF signal from P6.p activates the downstream components of the pathway, activating the transcription of the gene or set of genes necessary for proper VPC orientation. If either of these two events is compromised, the FGF pathway cannot direct the anterior orientation of P7.p.

Table 2. The sex myoblasts influence the polarity of P7.p

Genotype	M cell	% P-Rvl	*n*	*P*-value
lin-18(e620)	+	30	40	
lin-18(e620)	–	66	29	0.0065
sem-2(n1343); lin-18(e620)	–	68	40	0.0016
lin-18(e620); mig-2(RNAi)	Mig	56	50	0.0188
cwn-1(ok546); lin-18(e620)	+	26	100	
cwn-(ok546); lin-18(e620)	–	25	20	

Ablating the M cell enhances the phenotype of the *lin-18(e620)* single mutant, but does not enhance the *cwn-1(ok546); lin-18(e620)*, suggesting the SMs, which arise from the M cell, regulate vulval cell lineage polarity and CWN-1 is the necessary cue expressed in the SMs. *sem-2(n1343)* genetically ablates the SMs and *mig-2(RNAi)* causes a migratory defect in the SMs, denoted by Mig.

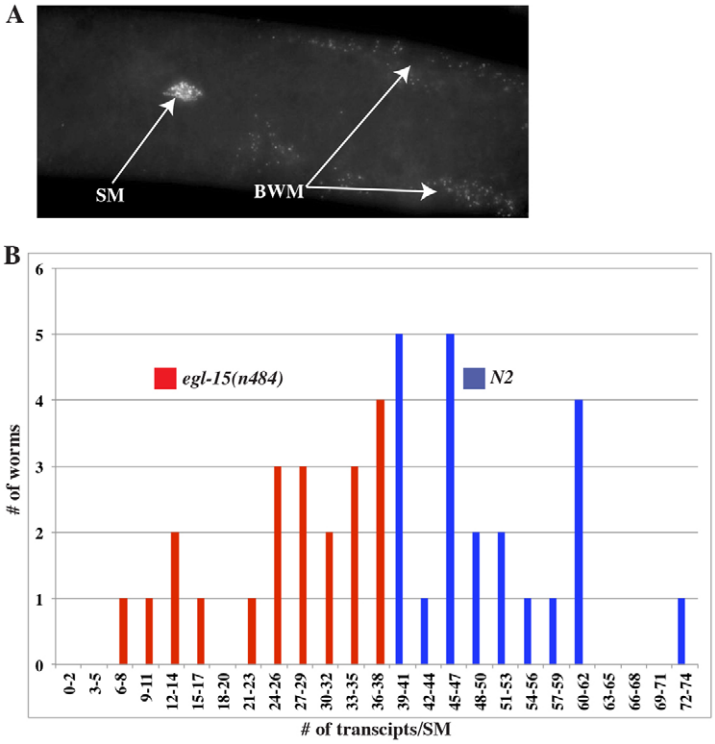

Fig. 7. FGF signaling regulates *cwn-1* expression in the SMs. (**A**) smFISH analysis of the *cwn-1* transcript in a wild-type worm at the time of the polarity decision. *cwn-1* is predominantly expressed in the posterior body wall muscle (BWM) and in the M cell/SM lineage. (**B**) A histogram quantifying the number of *cwn-1* transcripts per SM in wild-type (blue bars) and *egl-15(n484)* (red bars) backgrounds.

FGF signaling regulates expression of *cwn-1* in the sex myoblasts

The *C. elegans* genome encodes five different Wnt proteins, expressed in partially overlapping patterns across the anteroposterior axis, but only one, *cwn-1*, is expressed in the SMs (reviewed by Eisenmann, 2005; Harterink et al., 2011). Work in other animals has shown crosstalk between FGF and Wnt pathways, often leading to the regulation of Wnt by FGF (Hong et al., 2008; Stulberg et al., 2012; Yardley and García-Castro, 2012). We hypothesized that FGF signaling regulates a Wnt signal produced in the SMs that controls P7.p polarity. To test this idea directly, we used smFISH to quantify the number of mRNA transcripts of *cwn-1* found within the left and right SMs just prior to the polarity decision of P7.p in wild-type and reduced FGF signaling backgrounds (Fig. 7).

On average, the wild-type SMs each express 50 transcripts of *cwn-1* prior to the polarity choice of P7.p. In an *egl-15(n484)* background, the expression of *cwn-1* transcripts is reduced by ~50% on average with 23% of the SMs having one-third the number of wild-type transcript and 10% having as little as one-fifth of the number of wild-type transcripts. There is no overlap in SM transcript count between the wild-type and mutant backgrounds. The lowest wild-type SM transcript count is still greater than the highest SM transcript count in the mutant background: 40 transcripts per SM is the lowest wild-type count compared with 37 transcripts per SM for the highest *egl-15(n484)* count (Fig. 7; supplementary material Table S2). Therefore, FGF signaling regulates the expression of the Wnt ligand *cwn-1*. It cannot be determined just how much *cwn-1* transcript is needed to produce a wild-type vulval orientation, although previous work has examined how a change in transcript count affects phenotype (Raj et al., 2010). Examining the transcript count of *egl-15(n484)*, we hypothesize that the SMs with a higher *cwn-1* transcript count, similar to that of the wild type, produce a P7.p lineage with an anterior orientation. It is the SMs with a greatly reduced *cwn-1* transcript count that are likely to fall below the necessary threshold to orient P7.p to the anterior and, therefore, produce a P-Rvl phenotype.

cwn-1 acts instructively from both the anterior and posterior sides of P7.p

cwn-1 is expressed in the posterior body wall muscle and M cell descendants, making it the only Wnt ligand expressed from the anterior and posterior sides of P7.p during the polarity decision (Harterink et al., 2011) (also see Fig. 7). Previous work suggested that Wnt ligands instruct P7.p to orient towards the direction of the Wnt gradient: LIN-44 and MOM-2 towards the anterior and EGL-20 towards the posterior (Fig. 1). Genetic evidence indicates that *cwn-1* acts upstream of *lin-17*, a receptor necessary for the anterior signal (Gleason et al., 2006), and has been shown to bind to CAM-1, a receptor necessary for the posterior signal (Green et al., 2007). Because *cwn-1* is expressed on both sides of P7.p and has been shown to interact with receptors associated with the anterior and posterior pathways, we hypothesized that each gradient might instruct P7.p to orient towards the direction of the respective gradient. A *cwn-1* mutation had little effect on vulval orientation in a *lin-18* mutant [31% versus 26% P-Rvl in *lin-18(e620)* versus *cwn-1(ok546)*; *lin-18(e620)*, respectively].

All Wnts directing VPC polarity instruct the localization of SYS-1 to the P7.p daughter cell towards the gradient (Green et al., 2008). Despite being different Wnts, LIN-44 and MOM-2, acting through LIN-17 and LIN-18, respectively, both have the same molecular output of anterior SYS-1 localization. EGL-20, from the posterior, drives the posterior localization of SYS-1. We assume that each Wnt imparts a directional cue instructing SYS-1 to localize to the direction of the Wnt source. Therefore, CWN-1 from the SMs joins LIN-44 and MOM-2 in driving anterior localization, through an overall anterior Wnt gradient, and CWN-1 from the posterior body wall muscle joins EGL-20 in driving posterior localization, through an overall posterior Wnt gradient (Fig. 8). This assumption makes physical sense when considering mutations in FGF pathway

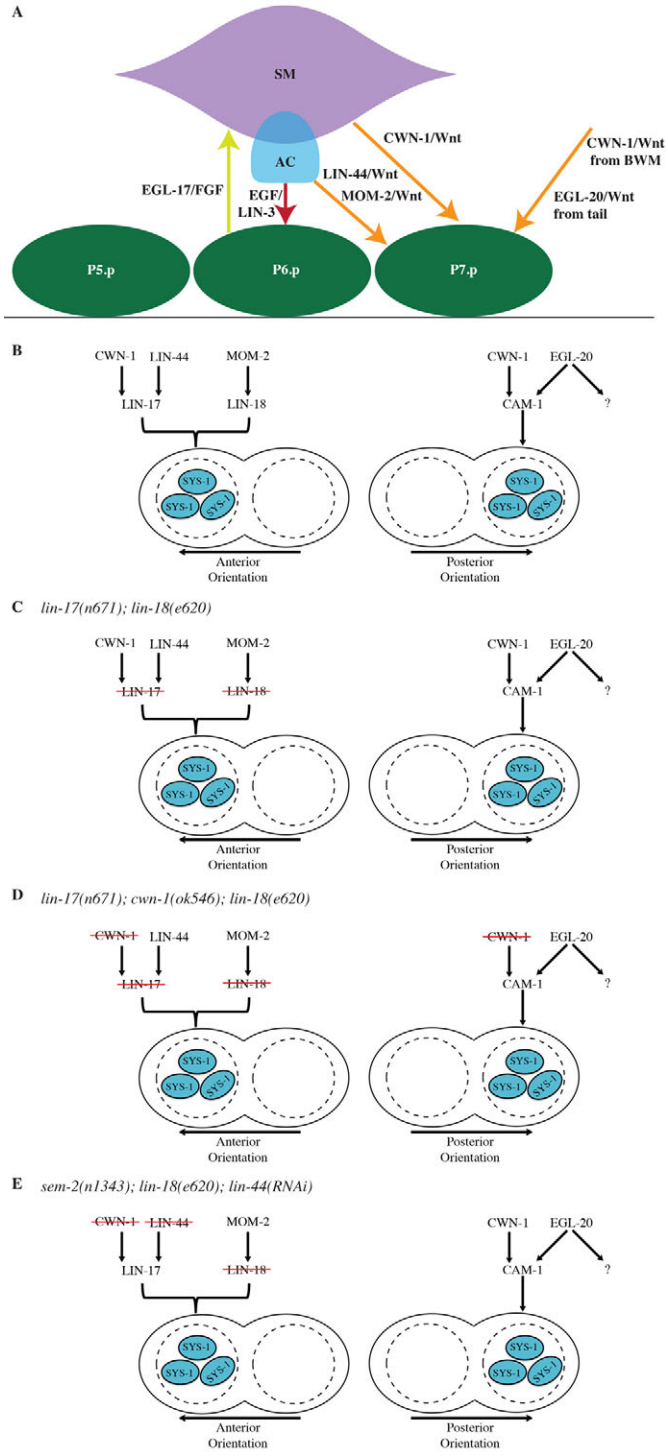

Fig. 8. The role of Wnt signaling on P7.p (**A**) The anchor cell (blue; AC) releases LIN-3 (red arrow), inducing the VPCs (green). Induction triggers the expression of the FGF ligand EGL-17 (yellow arrow) in P6.p, which activates the FGF pathway located in the SMs (purple). The FGF pathway regulates the expression of *cwn-1* in the SMs. The SMs are the anterior source of CWN-1 for P7.p whereas the posterior body wall muscle serves as the posterior source. All Wnt signals are depicted with an orange arrow. *lin-44* and *mom-2*, both expressed anterior to P7.p, express in the anchor cell whereas *egl-20* expresses in the tail, the posterior side of P7.p. (**B**) CWN-1 from the SMs and LIN-44 act through LIN-17, and MOM-2 acts through LIN-18. All three ligands act to drive SYS-1 localization to the anterior daughter of P7.p. Posterior-expressed CWN-1 and EGL-20 act through CAM-1 to drive SYS-1 localization to the posterior daughter of P7.p. Genetic data indicate that EGL-20 possibly acts through another, unknown receptor. (**C-E**) Examples of how mutations drive phenotypic output. In *lin-17(n671); lin-18(e620)*, all anterior receptors are eliminated resulting in a 100% P-Rvl phenotype. Mutations in *cwn-1* eliminate it from both sides of the pathway whereas *sem-2(n1343)*, owing to a genetic ablation of the SMs, eliminates only the anterior source of *cwn-1*.

components. The single mutants do not affect orientation because only one anterior Wnt is removed, leaving LIN-44 and MOM-2 to direct the localization of SYS-1. However in a *lin-18(e620)* double mutant, the animal has lost two anterior sources of Wnt, CWN-1 and MOM-2, and therefore the overall anterior Wnt gradient is greatly reduced allowing the posterior gradient to predominate. Likewise, if the posterior CWN-1 signal is compromised, the overall posterior Wnt gradient is reduced and SYS-1 is instructed to localize to the anterior daughter cell.

To test this hypothesis, we designed a construct that would provide an anterior gradient of CWN-1, namely *Pegl-17*::CWN-1::GFP, and therefore reinforce the anterior gradient. The *egl-17* promoter activates the expression of *cwn-1* in P6.p upon vulval induction (supplementary material Fig. S1). By expressing this construct in a *cwn-1(ok546); lin-18(e620)* background, the only source of CWN-1 comes from the anterior side of P7.p. Anterior-expressed CWN-1 suppresses the *cwn-1(ok546); lin-18(e620)* phenotype from 26 to 13% (P=0.1288). We hypothesized the P-Rvl phenotype of *cwn-1(ok546); lin-18(e620)* could be too mild at 26% to see the full suppression resulting from driving CWN-1 from the anterior, so we used a sensitized background that gives a higher initial P-Rvl phenotype. Treating *cwn-1(ok546); lin-18(e620)* worms with *lin-44* RNAi increases the percentage of P-Rvl to 52% owing to the role of LIN-44 acting upstream of LIN-17. Expressing the *Pegl-17*::CWN-1::GFP construct in *cwn-1(ok546); lin-18(e620)* worms treated with *lin-44* RNAi results in significant suppression of the P-Rvl phenotype to 30% (P=0.0210) (Table 3).

We next tested whether anterior CWN-1 could rescue the phenotype of *lin-18(e620) egl-15(n484)*, and found that it does rescue the phenotype from 63 to 38% (Table 3). We believe that the construct does not rescue fully back to 30% because in a *lin-18(e620) egl-15(n484)* animal the SMs are still producing a reduced CWN-1 signal from the posterior side of P7.p.

These data illustrate that CWN-1 provides an instructive anterior gradient sufficient to suppress the posterior gradient in the wild-type nematode (Table 3). If this cue were permissive, we would not expect to see a sole anterior source of CWN-1 suppress either *cwn-1(ok546); lin-18(e620), cwn-1(ok546); lin-18(e620)* grown in *lin-44* RNAi, or rescue the phenotype of *lin-18(e620) egl-15(n484)*. CWN-1, therefore, acts instructively from the anterior and posterior of P7.p. In the absence of a posterior signal, the anterior signal reinforces the progeny of P7.p to face the center and can suppress the P-Rvl phenotype. Likewise, in the absence of the anterior CWN-1 signal, through defects in the FGF pathway, or removal of P6.p or the SMs, the posterior signal instructs the progeny of P7.p to orient posteriorly when the anterior Wnt gradient has been compromised (Fig. 8; Table 4).

DISCUSSION

Our results describe an interaction between FGF and Wnt signaling in vulval cell lineage polarity. Through genetic analysis, we have shown that each component of the FGF pathway enhances the P-Rvl phenotype of LIN-18 mutants, but does not affect that of LIN-17,

Table 3. *cwn-1* acts instructively from the anterior and posterior sides of P7.p

Genotype	Anterior CWN-1 source	% P-Rvl	*n*	*P*-value
cwn-1(ok546); lin-18(e620)	–	26	100	
cwn-1(ok546); lin-18(e620)	+	13	45	0.1288
cwn-1(ok546); lin-18(e620) lin-44(RNAi)	–	52	61	
cwn-1(ok546); lin-18(e620) lin-44(RNAi)	+	30	50	0.0210
egl-15(n484) lin-18(e620)	–	63	52	
egl-15(n484) lin-18(e620)	+	38	40	0.0202

Driving CWN-1 from the anterior side of P7.p suppresses the P-Rvl phenotype of *cwn-1(ok546); lin-18(e620)* mildly and significantly suppresses the phenotype of *cwn-1(ok546); lin-18(e620)* grown in *lin-44* RNAi. Anterior expression rescues the phenotype of *lin-18(e620) egl-15(n484)*.

Table 4. Genetic data for anterior and posterior pathway components

	% P-Rvl	*n*
Anterior components		
lin-17(n671)	74	100
lin-18(e620)	31	100
lin-17(n671); lin-18(e620)	100	40
lin-18(e620); lin-44(RNAi)	70	56
lin-17(n671); mom-2(or42)	100	103
lin-18(e620) egl-15(n484)	63	52
sem-2(n1343); lin-18(e620)	68	40
sem-2(n1343); lin-18(e620); lin-44(RNAi)	82	45
Posterior components		
lin-17(n671); cam-1(gm122)	46	54
lin-17(n671); vang-1(ok1142)	48	60
lin-17(n671); egl-20(hu120)	6	52
egl-20(hu120); lin-18(e620)	8	51
lin-17(n671); egl-20(hu120); lin-18(e620)	50	52
lin-17(n671); cwn-1(ok546)	53	40
lin-17(n671); cwn-1(ok546); lin-18(e620)	92	47

Combinations of mutations for anterior pathway components increase the P-Rvl penetrance whereas mutations in posterior components suppress the P-Rvl phenotype. *cwn-1* is the only component found in both pathways.

indicating a specific interaction between FGF and LIN-17, probably CWN-1 acting on LIN-17 but not LIN-18. The underlying mechanisms of the P-Rvl phenotype can be seen on the molecular level through the localization of the β-catenin ortholog SYS-1. FGF signaling indirectly controls the localization of SYS-1 to the anterior daughter cell of P7.p, which leads to the wild-type vulval orientation. FGF signaling does not directly influence the vulval lineage orientation, but instead is required for the regulation of CWN-1 expression, which acts instructively from both sides of P7.p (Fig. 8; Table 4). CWN-1 is the only Wnt ligand expressed on the anterior and posterior of P7.p at the time of its polarity decision and acts upstream of receptors involved in directing P7.p to face the anterior and posterior: LIN-17 and CAM-1, respectively.

How does P7.p always orient towards the anterior in the wild-type worm? Genetic data suggest that MOM-2 and LIN-44 have a greater ability to direct the anterior orientation of P7.p, with CWN-1 acting as a minor player. Both posterior-expressed CWN-1 and EGL-20 act over a distance and form a posterior-anterior gradient that has the ability to direct the orientation of P7.p towards the posterior, though the concentration of posterior Wnts might be much lower compared with anterior-expressed Wnts by the time they reach the VPCs (Coudreuse et al., 2006). Expressing either CWN-1 or EGL-20 from the anterior of P7.p (from the anchor cell or P6.p) is sufficient to redirect the orientation of P7.p towards the anterior. All four Wnts involved in vulval orientation direct the localization of SYS-1 despite acting through three different receptors, all of which are present in the same cell, P7.p. There is receptor specificity, but all Wnts seem to have the same effect: P7.p orients in the direction of the highest Wnt gradient. P7.p always faces the anterior in a wild-type worm because of the three anterior sources of Wnts in close proximity to P7.p. Only by removing these sources can we begin to see the effects of the posterior Wnts; these same posterior Wnts can impart an anterior-directing cue when repositioned. The two posterior Wnts EGL-20 and CWN-1 both activate competence to respond to LIN-3 in the anterior VPCs and may have the same molecular activity (Pénigault and Félix, 2011). A possible hallmark of Wnt-mediated patterning within *C. elegans* could be similar molecular outputs from genes that are not truly redundant.

How similar is Wnt-driven VPC patterning to other systems? A major difference between *C. elegans* and *Drosophila* is that no Wnts have been implicated in *Drosophila* planar cell polarity whereas Wnts play a major role in patterning the VPCs. By contrast, the receptor CAM-1/Ror and the transmembrane protein VANG-1/Van Gogh, antagonize LIN-17 and LIN-18 by directing the localization of SYS-1 to the posterior daughter of P7.p. The antagonism between Fz and Van Gogh is a hallmark of planar cell polarity in the *Drosophila* wing (Seifert and Mlodzik, 2007; Gao, 2012; Singh and Mlodzik, 2012), but much less is understood about the interaction between Ror and Van Gogh (Gao et al., 2011).

Other comparisons can be drawn between *C. elegans* and vertebrate Wnt signaling. Wnts LIN-44 and CWN-1 act through LIN-17/Fz and MOM-2 acts through LIN-18/Ryk to direct SYS-1 to localize to the anterior daughter of P7.p. Although the possibility of a Fz-Ryk co-receptor complex exists in the mammalian systems (Lu et al., 2004), LIN-17 and LIN-18 function in parallel pathways despite both directing the localization of SYS-1. Recent work in vertebrates has shown FGF regulates the expression of Wnt in a manner similar our observations in *C. elegans* vulval patterning. FGF regulates the expression of Wnt in the non-neural ectoderm of the chick (Yardley and García-Castro, 2012). FGF also elevates Wnt expression, through inhibition of Wnt antagonists, in the zebrafish tailbud (Stulberg et al., 2012). Furthermore, our results illustrate a network of signals, relayed back and forth between different tissues: the gonadal anchor cell expresses an EGF signal that induces the ectodermal vulval cells, activating an FGF signal that is sent to the mesodermal sex myoblasts, which enables the regulation of a Wnt that directs the patterning of the ectodermal vulval cells. This relay between different tissues bears resemblance to *Xenopus* in which it has been shown that Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm, which then directs neural crest formation in the overlying ectoderm (Hong et al., 2008).

Using the *C. elegans* vulva as a model, we have shown that a network of Wnt signals, with distinct receptor specificity, direct the orientation of the vulval precursor cells through the localization of β-catenin. One of these Wnts, CWN-1, is regulated through the activity of the FGF pathway in a crosstalk between multiple tissues that enables the efficacy of its directional cue.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
P.J.M., A.A. and P.W.S. conceived the experiments. P.J.M. carried out all the experiments with help from T.-F.H. and C.H.S. for the FISH experiments. P.J.M. and P.W.S. wrote the manuscript.

Supplementary material
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